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REGULAR ARTICLE

Omentum acts as a regulatory organ controlling skeletal muscle repair of mdx mice diaphragm

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Abstract

Duchenne muscular dystrophy is a lethal X-linked muscle wasting disease due to mutations of the dystrophin gene leading to distinct susceptibility to degeneration and fibrosis among skeletal muscles. This study aims at verifying whether intense mdx diaphragm remodeling could be attributed to influences from the omentum, a lymphohematopoietic tissue rich in progenitor cells and trophic factors. Mdx omentum produces growth factors HGF and FGF and increased amounts of VEGF with pleiotropic actions upon muscular progenitors and myoblast differentiation. Histology revealed that the absence of the omentum reduced inflammation and collagen deposition in the diaphragm. The diaphragm from omentectomized mdx mice presents impaired repair with a predominance of collagen type I deposition, decreased muscle regeneration and a reduction in collagen type IV and indication of altered basal lamina integrity in the diaphragm. Omentectomy further reduced inflammatory infiltration and NFκ-B activation but a change in the pattern of muscle inflammation with low numbers of the $F4/80^{+}CD206^{+}$ M-2 macrophage subset. Although omentectomized mice had high levels of Pax7, myogenin and TNF-α, the percentage of myofibers undergoing regeneration was low thus suggesting that a lack of the omentum halts the muscle differentiation program. Such results support that omentum exerts a regulatory function inducing an inflammatory process that favors regeneration and inhibits fibrosis selectively in the diaphragm muscle thus being a potential site for therapeutic interventions in DMD.

Keywords Omentum · mdx · Macrophages · Fibrosis · Diaphragm · Muscle regeneration

Douglas Florindo Pinheiro and Rafael Ferreira da Silva contributed equally to this work.

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Introduction

Duchenne muscular dystrophy (DMD) is a devastating X-linked wasting muscle disease that affects 1:5000 births of boys in the world (Mendell et al. 2012). It is caused by mutations leading to the absence of dystrophin, a 350 kDa protein that anchors the sarcolemma to the extracellular matrix (Johnson et al. 2013). In the absence of dystrophin, muscle fibers present deregulation of calcium influx (Allen et al. 2010) and decreased NO production being more vulnerable to degeneration and alteration in the vascular tonus (Loufrani et al. 2004) during the stress of muscle contraction. Moreover, abnormal Golgi complex function, leading to glycosylation defects of extracellular matrix molecules, contributes to the loss of muscle fiber integrity (Sharpe et al. 2013). The mdx mouse model displays the same genetic defect as several human DMD patients (McGreevy et al. 2015) but presents efficient muscle tissue remodeling despite severe cycles of muscle degeneration and a slight reduction in total lifespan (Chamberlain et al. 2007). The ability of mdx mice to compensate for the lack of dystrophin probably relies on efficient myofiber type reorganization (Selsby et al. 2012) partly due to the expression of smaller forms of dystrophin (Pigozzo et al. 2013) and reactivation of utrophin expression, the embryonic form of dystrophin (Weir et al. 2004).

Muscles are not equally susceptible to degeneration and the exact mechanisms governing muscle degeneration and fibrosis remain largely elusive. The diaphragm is the most severely affected muscle with intense fibrosis and continuous inflammation (Moens et al. 1993; Bani et al. 2008). Moreover, inflammatory genes are upregulated in some muscles before the onset of muscle degeneration, suggesting that mechanical injury might be a secondary element in the muscular lesion. Likewise, macrophage infiltration is a decisive element defining the outcome of a muscle lesion. Inflammatory macrophage M-1 subset is important in the early phase of muscle lesion by promoting inflammation and clearance of damage-associated molecular pattern molecules (DAMPs), while the regulatory macrophage (M-2) subset controls the inflammatory response and promotes muscle repair (Tidball and Wehling-Henricks 2007; Arnold et al. 2007).

It is conceivable that the insertion of the diaphragm in the peritoneum suggests the coelomic-associated lymphoid tissues (CALT) as main players activated by danger signals (DAMPs) derived from the muscle injury. Previously, we showed that the omentum, a lymphohematopoietic organ residing in the peritoneal cavity, is activated in mdx mice (Pinheiro et al. 2012). The omentum controls immune responses in the peritoneum, displays high numbers of B-1 lymphocytes and has a different ontogenesis from other secondary lymphoid organs (Ansel et al. 2002; Rangel-Moreno et al. 2009; Meza-Perez and Randall 2017). Additionally, omentum harbors functional hematopoietic and mesenchymal progenitors in adipose and mesothelial areas and produces CXCL2 and LIF cytokines that are involved in the attraction of stem cells and in the maintenance of the stemness respectively. The omentum is able to sustain full hematopoiesis and enhances tissue regeneration in response to organ damage by providing precursor cells and tissue-specific growth factors (Hirai et al. 1994; Pinho et al. 2005; Litbarg et al. 2007; Singh et al. 2008). In the present work, we sought to determine the influence of omentum in the inflammatory infiltrate and tissue remodeling profile of the diaphragm in the mdx mouse by analyzing the effects of surgical removal of the omentum.

Material and methods

Animals

The study protocol and handling of animals were approved by the Institutional Animal Care Committee CEUA UFF (protocol no. 171, 717 and 1054) and conducted according to the Conselho Nacional de Controle de Experimentação Animal, CONCEA. Male mdx dystrophic and age-matched control C57BL/10J non-dystrophic mice at age of 12 and 24 weeks were included in this study. Both strains were maintained in the animal housing facilities at the Instituto de Biologia in the Universidade Federal Fluminense at a constant temperature (20 °C) with a light to dark cycle of 12:12 h and received acidified water and a commercial rodent diet ad libitum.

Omentectomy

This procedure was performed on mice at 4 weeks (w) of age as previously described (Berberich et al. 2008). Briefly, after intraperitoneal injection with ketamine (100 mg/kg) and xylazine (10 mg/kg), an incision in the upper left corner of the abdomen was made and the intact omentum was carefully removed. For SHAM controls, a superficial skin incision was made only to induce stress. Mice were sacrificed at 12 and 24 weeks old corresponding to main phases of regeneration and fibrosis.

Histochemistry

The gastrocnemius muscle, diaphragm and omentum from 12 and 24-week-old mice were carefully removed and fixed in formalin-buffered Millonig fixative (pH 7.2) for 24 h. Sections (5-μm thick) of tissue embedded in paraplast (Sigma, St. Louis, Mo., USA) were stained with Sirius Red for analysis of histological alterations. Areas stained in red were quantified as collagen deposition and regenerating fibers were identified by strong basophilic and centrally-located nuclei (Dorchies et al. 2013). For quantification, high-definition whole-area images of all cross-sections from a single mdx

mouse at each time point were obtained from individual photomicrographs with a microdigital camera mounted on a Leica DMI300B (Mannheim, Germany) with a × 10 objective. Images were mounted with Photomerge from Adobe Photoshop CS3 Extended software. The total area occupied by regenerating myofibers and collagen deposition was determined with ImageJ® (National Institutes of Health, NIH). Results were expressed as the percentage of the total area in the cross-sections. Each experimental group was composed of at least five animals and three slices were analyzed per animal. For total collagen analysis, sections stained by Sirius Red were analyzed by light microscopy and the total collagen area was quantified and expressed as a percentage of the area of the total tissue section. For collagen subtype analysis, images were taken using dark-field polarized light to evidenced collagen I (red), collagen III (green) and collagen IV (yellow). The images obtained were analyzed by ImageJ® software using the hue component (red 2–9/230–256, green 52–128 and yellow 39–51) obtained from the 8-bit hue images that contained 256 possible colors. The number of pixels within each hue range was expressed as a percentage of the total number of collagen pixels (Cuttle et al. 2005; Balachandran et al. 2006; Lees et al. 2013). Each experimental group was composed of at least four animals and three slices with 150-μm interval levels were analyzed per animal. The results for each collagen subtype were expressed as a percentage of the total collagen area.

Immunohistochemistry

Omentum from mdx and control mice at age of 12 weeks and 24 weeks was embedded in OCT (Tissue-TEK, Elkhard, Ind., USA). Sections (5-μm thick) were mounted on poly-l-lysine pre-coated slides, fixed in acetone and blocked for endogenous peroxidase activity with 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 5 min. Immunostaining was carried out by an initial incubation with rabbit polyclonal anti-VEGF, anti-HGF and anti-SDF-1 antibodies (Santa Cruz Biotechnology, CA, USA). The optimal concentration of the specific primary antibody diluted in PBS (pH 7.4) was applied and then followed by incubation at room temperature in a moist chamber for 60 min. Sections were further incubated for 40 min with biotinylated anti-rabbit antibody (Invitrogen, California, EUA) and with streptavidin-peroxidase complex (Sigma) followed by extensive washes with PBS. Enzyme activity was revealed with aminoethyl carbazole (Sigma) in the presence of hydrogen peroxide and counterstained with Mayer's hematoxylin (Sigma). For histological studies, at least three animals were examined.

For the macrophage analysis, frozen diaphragm slices were incubated with biotinylated rabbit anti-F4/80 macrophage marker and a rat anti-CD206, a lectin complement receptor that is present in regulatory M-2 macrophages; both were purchased from Serotec (Oxford, UK). After washing with PBS, slides were incubated with streptavidin Cy3 (Molecular Probe, USA) and anti-rat Alexa 488 (E-bioscience). Images were acquired with a fluorescence microscope (Leica® DMI3000).

Real-time PCR

Whole-muscle or omentum was homogenized in TRIzol. Total RNA from diaphragm muscles was isolated using commercially available kit Direct-zol™ RNA MiniPrep (Zymo Research, Irvine, CA - EUA), according to the manufacturer's instructions. In order to evaluate the relative expression of TNF-α, myogenin and VEGF, qualitative real time was performed using the TaqMan® (Applied Biosystems©) system according to manufacturer instructions. Briefly, 4 μL of cDNA was mixed with 6 μL of the Taqman mastermix and 0 .6μL of the corresponding TaqMan® probe (see Table 1). Ideal thermocycling was applied using a StepOne™ Real-Time PCR System (Applied Biosystems©). The mRNA levels of the target genes were assessed using the "relative quantification" methodology using the 2-ΔΔCt formula. Data from the test samples ($n = 3$) were normalized against the mRNA levels detected for the beta-actin gene and expressed as fold change compared with SHAM samples.

Quantitative real-time PCR for PAX7 gene was performed with 2 μl of cDNA (10% of final volume) using the SYBR® Green Master Mix (Applied Biosystems™) on StepOne™ real-time PCR system (Applied Biosystems™). The cycling parameters used for all primers were as follows: 95 °C for 10 min and PCR 45 cycles of 30 s at 95 °C for denaturation, 1 min at 60 °C for annealing and 30 s at 72 °C for extension. RNA and cDNA were obtained as previously described. Quantification was normalized according to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer sequences were as follows: Pax7 (132 bp): Forward 5′-tctccaagattctgtgccgat-3′; Reverse 5′-cggggttctctctcttatactcc-3′ and GAPDH (245 bp): Forward 5′-ggtggaggtcggtgtgaacgga-3′; Reverse 5′-tgttagtggggtctcgctcctg-3′. The data were obtained from analysis in the StepOne™ Software V2.3 (Applied Biosystems). For real-time PCR, three animals were examined.

Table 1 Primers used in real-time reaction

Genes	Applied code	Stain	Size (bp)
VEGFα	Mm01281449_m1	FAM/MGB	81
TNF-α	Mm00443258-m1	FAM/MGB	81
Myogenin	Mm00446195-g1	FAM/MGB	89
Beta-actin	Mm00607939-S1	FAM/MGB	115

Western blotting

Diaphragm muscles were homogenized in a glass homogenizer with protease inhibitor buffer (Sigma, St Louis, MI). Protein extracts were clarified by centrifugation at 12,000×*g* for 15 min at 4 °C. Protein quantification was determined by the Lowry method (Lowry et al. 1951) and the concentration adjusted with buffer pH 6.8 (173 mM Tris, 30% glycerol, 3% sodium dodecyl sulfate, 3% β-mercaptoethanol, 0.1% bromophenol blue). Samples were denatured by boiling for 5 min and 40 μg of protein was loaded per well on 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Sample electrophoresis was carried out at 90 V for 2 h at 4 °C. The separated proteins were transferred to PVDF membranes (Hybond-P; Amersham Biosciences, Fairfield, CT) and further blocked with 5% nonfat dry milk and 1% bovine serum albumin in 0.05% Tween-20 Tris-buffered saline (TBST) pH 7.4 for 2 h on a rocking platform at room temperature. Thereafter, membranes were incubated with the specific primary antibodies: mouse monoclonal anti-TNF-α (at 1:700 dilution; Peprotech, USA) and rabbit polyclonal anti-NFκB p65 (at 1:100 dilution; Santa Cruz, USA) in 5% nonfat dry milk in TBST at 4 °C overnight. After washing three times for 10 min with TBST, blots were incubated for 2 h with anti-mouse and anti-rabbit peroxidase-conjugated secondary antibodies (both Cell Signaling, USA). Blots were washed three times in TBST and bands were identified using ECL Plus (Amersham Biosciences, Fairfield, CT) for chemiluminescent detection and subsequent film exposure for 5 min. The presence of NFκB p65 and TNF-α was verified by comparing protein bands to the Molecular Rainbow Weight Marker (Amersham Biosciences; Fairfield, CT). Same procedures were performed using rabbit anti-mouse Tubulin α (Cell Signaling, USA) as a loading control.

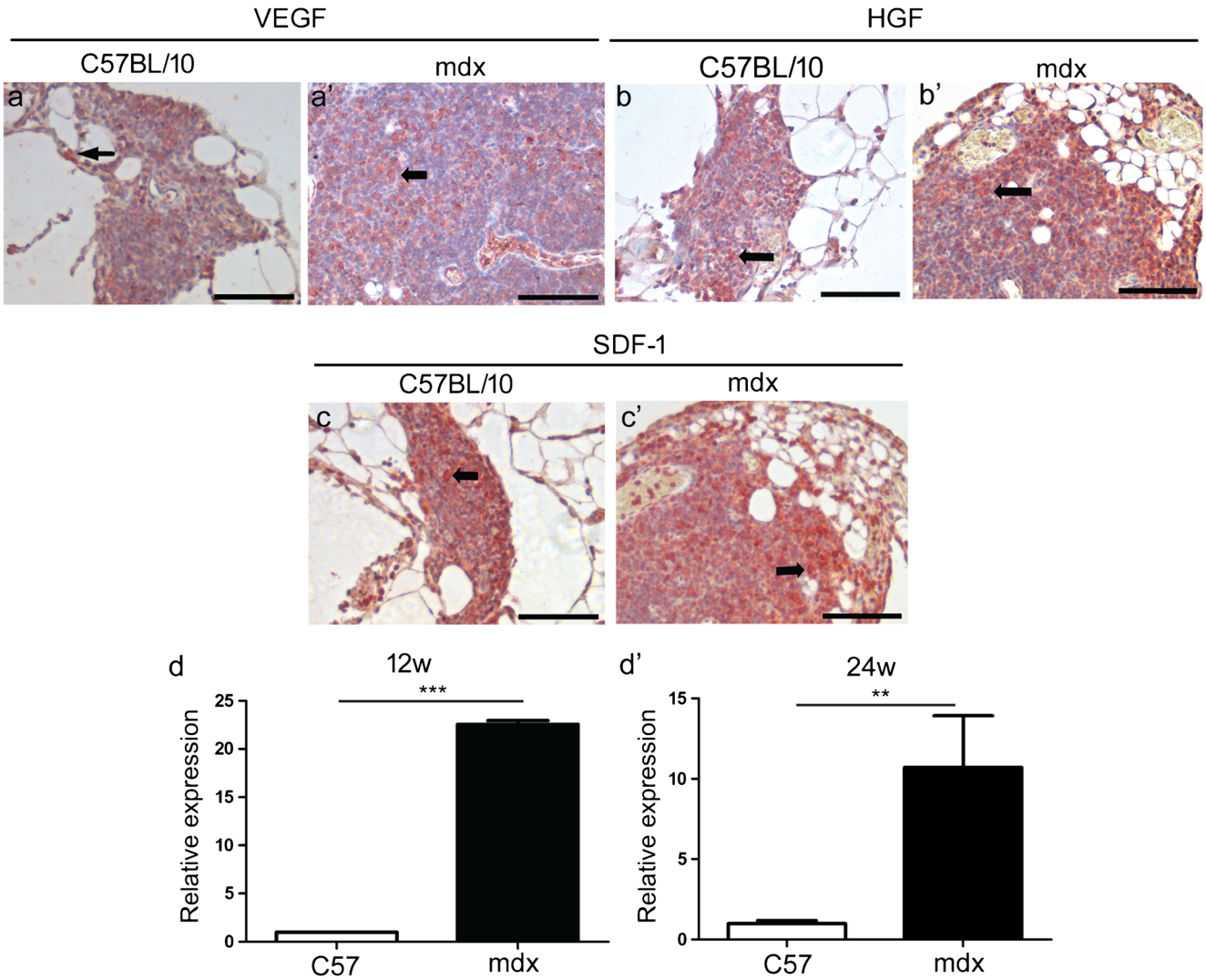

Fig. 1 Omentum produces VEGF, SDF-1 and HGF. Omentum from 24-week-old C57 (a, b, c) and mdx (a', b', c') mice were harvested and frozen sections were immunolabeled for VEGF (a, a'), HGF (b, b') and SDF-1 (c, c'). Bar = 50 μm. Real-time PCR confirms VEGF results by mRNA expression at 12 (d) and 24 (d') weeks. $^{**}p < 0.005$, $^{***}p < 0.001$

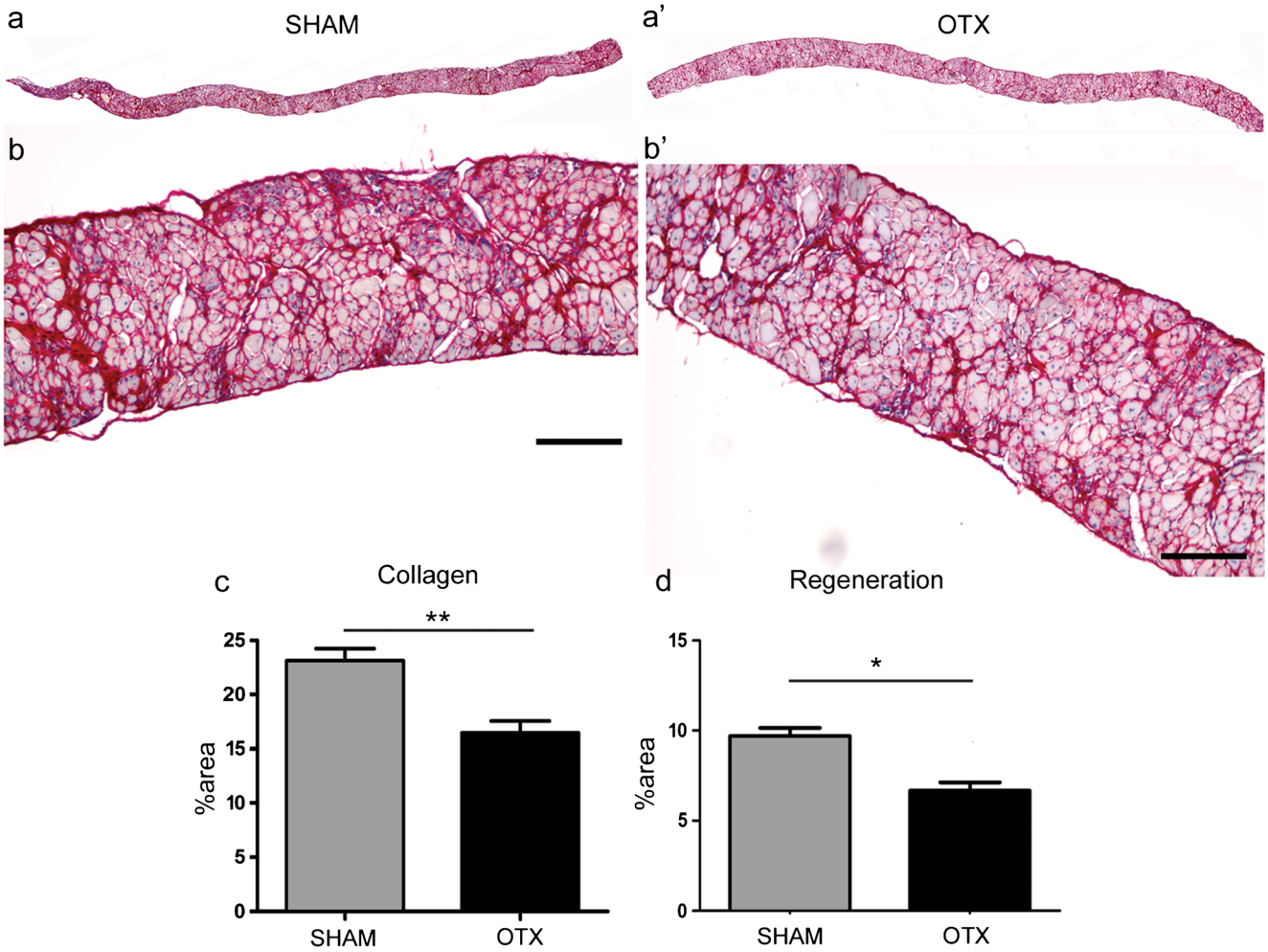

Fig. 2 Omentectomy decreases regeneration and collagen deposition in the mdx diaphragm at 24 weeks old. Sirius Red staining of the diaphragm with a photomerge representation (a, a') and representative micrograph (b, b') from sham (a, b) and OTX (a', b') mdx mice at 24 weeks old. Histomorphometric analysis reveals decreased collagen deposition (c) and regeneration (d) in the diaphragm of OTX mice. Bars = 100 μm. (*$p < 0.05$, **$p < 0.005$)

Statistical analysis

GraphPad Prism Version 5.0 was used for calculating means and standard deviations, building graphs and performing statistical tests. For comparison of two individual sets of data, a two-tailed unpaired *t* test was used. The coding used for the level of significance was * for $p < 0.05$, ** for $p < 0.005$ and *** for $p < 0.001$.

Results

Omentum of mdx and C57BL/10 mice produce VEGF, HGF and SDF-1

VEGF is a key growth factor implicated in the migration and survival of muscle progenitors. We performed immunohistochemistry for the detection of VEGF in the adipose tissue, mesothelium and milky spot areas from mdx and C57 omentum. A higher amount of VEGF positive cells was observed in the pleural milky spot of mdx mice in comparison to the C57 control (Fig. 1a). This finding was confirmed by real-time PCR that indicated a 20- and 10-fold increase of VEGF expression in mdx of 12 and 24 weeks of age in comparison to control groups ($p < 0.005$ and 0.001 respectively, Fig. 1d). Moreover, intense immunostaining for HGF, a factor that stimulates satellite cell activation (Tatsumi et al. 1998) and SDF-1, a pivotal player in stem cell activation and migration, including muscle progenitors (Kowalski et al. 2016) in vessels and mesothelium, was also observed although major staining was concentrated within the milk spot areas (Fig. 1b, c).

Omentectomy induces fibrosis and decreased regeneration

We observed previously that Omentectomy (OTX) diminished muscle regeneration, macrophage infiltration and total

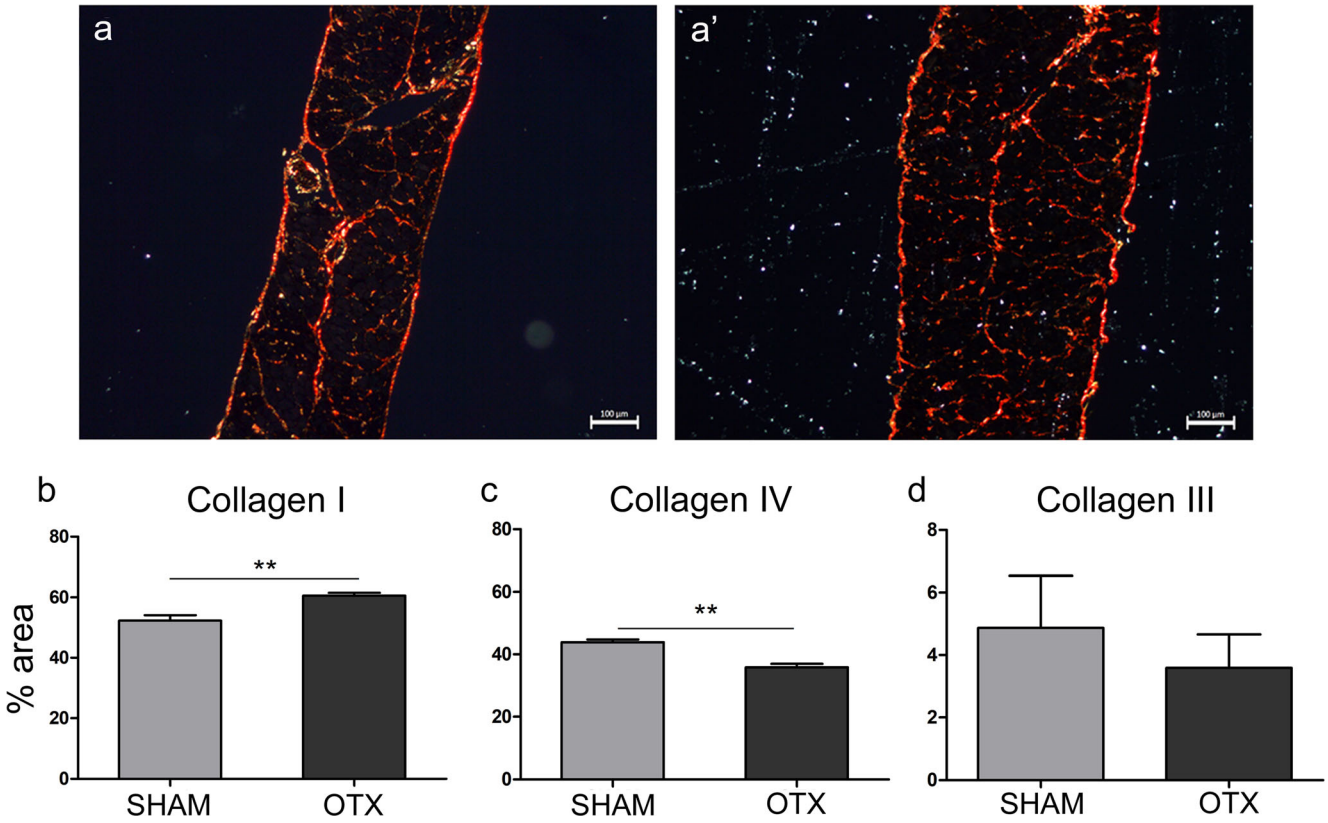

Fig. 3 Omentectomy increased collagen I deposition and reduced collagen IV in the diaphragm of mdx mice at 24 weeks. Polarized light analysis of the collagen type distribution in the diaphragm of SHAM (a) and OTX mdx mice (a'). Histomorphometric analysis of collagen I (b), collagen IV (c) and collagen III (d). Bars = 100 μm. $^{**}p < 0.005$

collagen deposition in mdx mice at 12 weeks of age (Pinheiro et al. 2012). Herein, we also found a reduction of 28.3% ($p < 0.005$) in collagen deposition and 45% ($p < 0.05$) in muscle regeneration of the mdx diaphragm at 24 weeks (Fig. 2). Interestingly, the gastrocnemius muscle from OTX mdx mice showed no significant difference in regeneration and collagen deposition at 12 and 24 weeks in relation to sham-operated mice (Fig. S1). This result indicates that the omentum exerts relevant effect especially on the diaphragm muscle and further highlights the peritoneum as a likely site for selectively controlling the pathology of the diaphragm.

To better define the putative influence of the omentum upon collagen deposition in the diaphragm of OTX mdx mice, we analyzed Sirius Red staining with a polarized light microscope. Compared with age-matched sham-operated mdx mice, the OTX mdx mice had a high content of collagen I ($p < 0.005$) but less collagen IV ($p < 0.005$, Fig. 3). Collagen I deposition is elicited upon the stimulation of myofibroblasts by cytokines (e.g., TGF-β and IL-13) as an indication that regenerative repair was unattainable. Therefore, we conclude that the omentum plays a decisive role in inhibiting muscle scarring. As collagen IV is a major component of the myofiber basal lamina critical for niche settlement of satellite cells, we conclude that OTX also affected myofiber integrity and regeneration potential.

Omentectomy induces a reduction of M2 macrophage in the mdx diaphragm

The diaphragm stomata directly open into the peritoneum allowing an influx of peritoneal cells into the muscle especially when activated by inflammatory cytokines (Michailova 2001). Macrophages derived from the CALT are the main immune cell type resident within the peritoneal cavity. Confirming previous results (Pinheiro et al. 2012), we found a slight decrease of F4/80+ cells in the diaphragm from OTX mdx (Fig. 4a, a', e) at 12 weeks. To determine if OTX influences macrophage phenotype in the mdx diaphragm, we used anti-CD206, a lectin receptor characteristic of the M2 phenotype (Nawaz et al. 2017). We observed that this reduction was mainly caused by a decrease (around 1.5 times) in the numbers of M2 macrophage ($F4/80^{+}CD206^{+}$), a population with strong label intensity for the lectin CD206 cell marker (Fig. 4 b, b', e"). Recently, it was shown that satellite cells express a low CD206 intensity cell marker whereas high CD206 intensity can be found in dendritic and endothelial cells (Kosmac et al. 2018). Interestingly, we found a sharp decrease ($p < 0.05$) of $F4/80^{-}CD206^{+}$ cells (Fig. 4e"'). These results suggest that OTX worsens the overall remodeling of the diaphragm muscle by decreasing M2 macrophages, a phenotype associated with muscle regeneration.

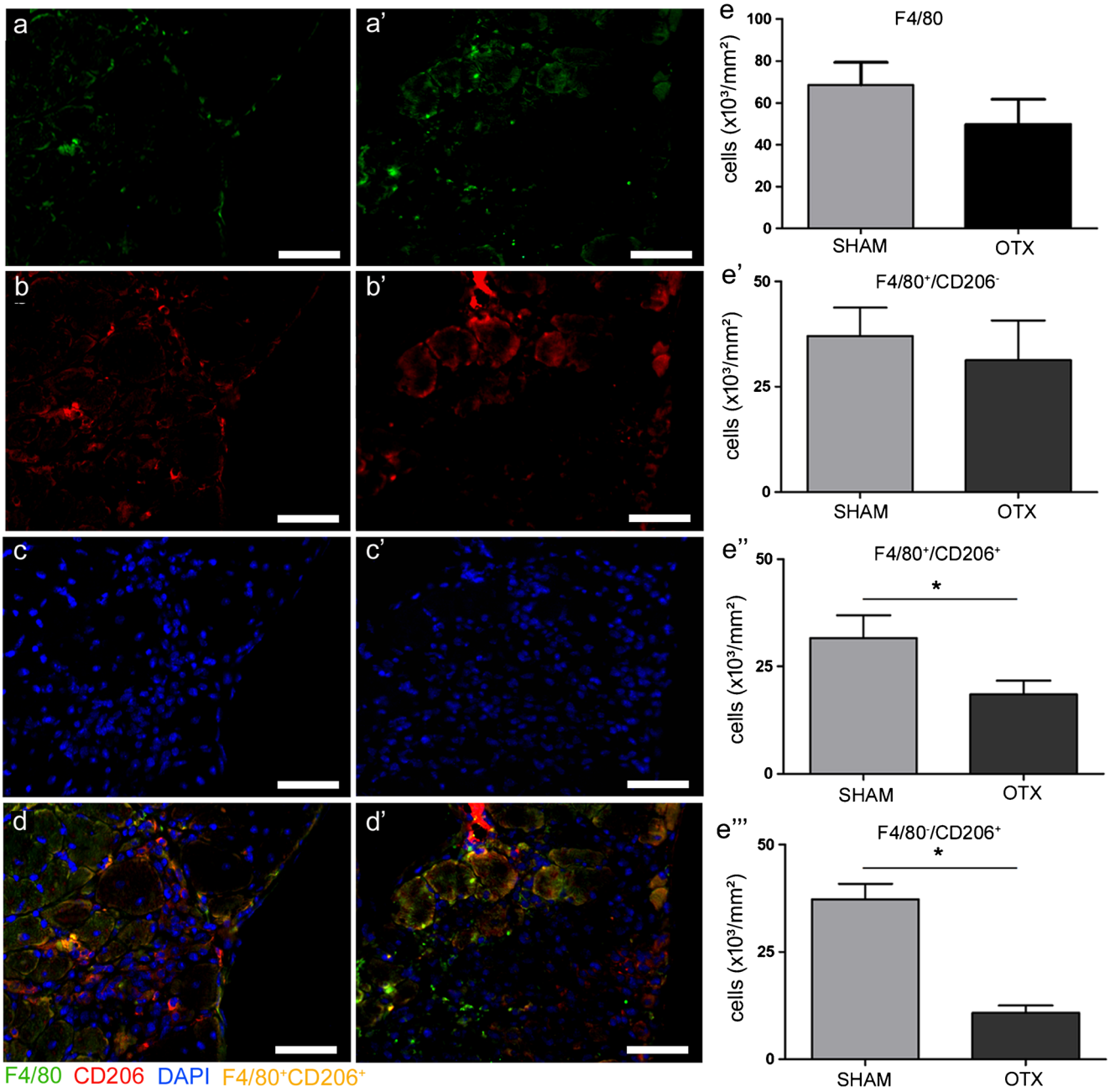

Fig. 4 Omentectomy reduced M2 macrophages in the OTX mdx diaphragm at 12 weeks. Omentum was surgically removed from 4-week-old mdx mice and euthanized at 12 weeks old corresponding to the main phase of muscle regeneration and the diaphragm was harvested for immunofluorescence. F4/80 (green) and CD206 (red) positive cells were analyzed in SHAM (a–d) and OTX mice (a'–d'). Histomorphometric analysis of the F4/80+ (e), F4/80^{+}CD206^{-} (e'), F4/80^{+}CD206^{+} (e") and F4/80^{-}CD206^{+} (e"') cell subtypes. Bars = 50 μm (*$p < 0.05$)

Modulation of inflammation and muscular regeneration in diaphragm from OTX mdx

We further evaluated the effect of OTX upon the expression of molecules related to inflammation and muscular regeneration. We observed that OTX mdx mice had decreased ($p < 0.05$) levels of NFκB transcription factor (Fig. 5a, b) possibly due to low numbers of macrophages in the inflammatory infiltrate. We observed an increase of TNF-α mRNA ($p < 0.05$) and protein (not significant) in the OTX diaphragm of mdx mice in comparison with the SHAM group (Fig. 5a–e'). However, detection of TNF-α protein was examined only in mice at 12-week old, i.e., during the less pronounced TNF-α mRNA increase. In addition, OTX diaphragm at 12 weeks showed an increase of PAX7 (Fig. 5d), a transcription factor present in satellite cells. Since TNF-α upregulation could be enhancing myoblast activation and differentiation, it was further important to verify myogenin expression in OTX mice. We found a significant increase of

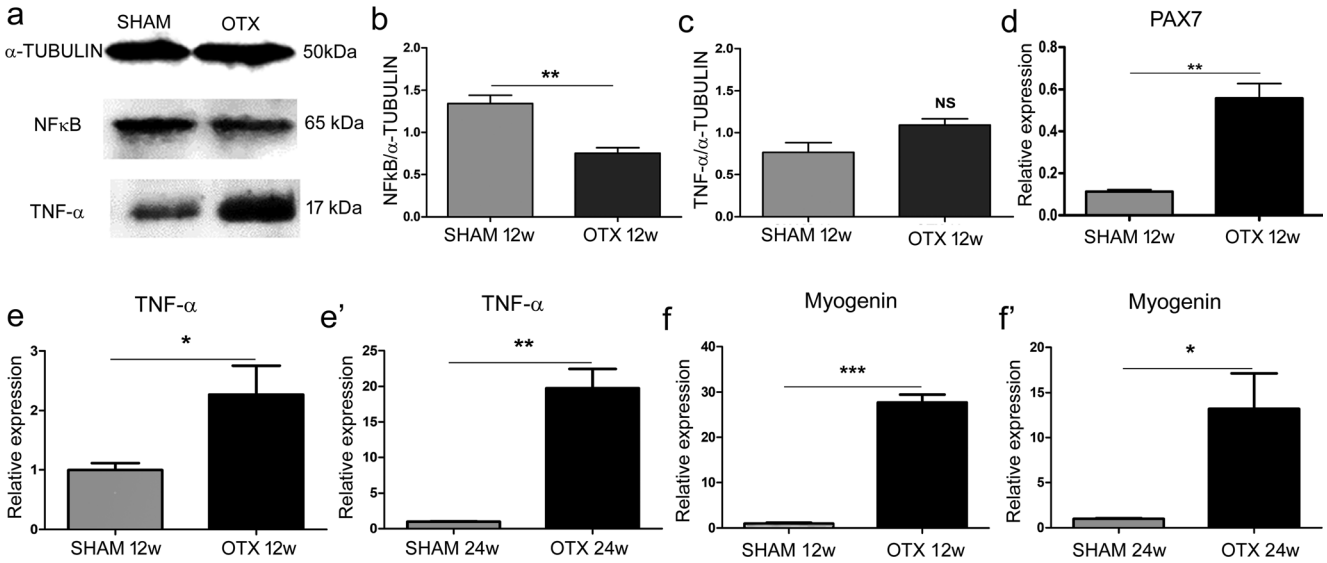

Fig. 5 Omentectomy changes the inflammatory pattern and muscular regeneration in mdx mice. Omentum was surgically removed from 4-week-old mdx mice and mice were euthanized at 12 and 24 weeks old corresponding to main phases of regeneration and fibrosis respectively. Representative bands in western blot gel showing tubulin loading control, NFκB and TNF-α (a) and relative densitometry analysis of NFκB (b) and TNF-α (c); measurements of PAX7 (d) by real-time PCR show increased mRNA levels in SHAM mdx diaphragms at 12 weeks. The data were normalized against the mRNA levels detected for the glyceraldehyde 3-posphate dehydrogenase (GAPDH). Measurements of TNF-α (e) and myogenin (f) by real-time PCR showed that mRNA levels were elevated in OTX mdx diaphragm at 12 (e, f) and 24 weeks old (e', f'). Test samples were normalized against the mRNA levels detected for the beta-actin gene and expressed as fold change compared to SHAM. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$

myogenin mRNA in the OTX diaphragm at 12 and 24 weeks when compared with sham-operated (Fig. 5f, f'), thus pointing to an increase in the myoblast differentiation.

Discussion

Cardiorespiratory insufficiency is still the major cause of death in DMD in spite of all advances in supportive therapy (Kieny et al. 2013; Van Ruiten et al. 2016) likely due to diaphragm failure, as this muscle is the most affected by the lack of dystrophin. The causative factors leading to alterations in the diaphragm in DMD patients and in the mdx mice remain elusive. We have previously shown that the peritoneal environment and the omentum are activated in mdx mice, which has a direct influence on diaphragm inflammation and remodeling (Pinheiro et al. 2012). In the present work, we sought to further characterize the influence of the omentum in the mdx diaphragm. The omentum is a lymphohematopoietic organ with long-described healing properties and capable to prevent sepsis due to intestinal perforations (Liebermann-Meffert 2000). Additionally, the transposition of the omentum has been used in clinics for the treatment of many conditions, including abdominal organ damage, spinal cord injuries and even Alzheimer's disease (Goldsmith 2007, 2009; Singh et al. 2009). Upon activation, the milk spot area expands and the hematopoietic activity increases concomitantly with the number of stem cells expressing embryonic and adult markers (e.g., CD34, SSEA, OCT-4, NANOG and CXCR4) (Litbarg et al. 2007; Singh et al. 2008). Moreover, mesenchymal stem cells of the omentum also improve cardiomyocyte differentiation and enhance survival (De Siena et al. 2010) highlighting the ability of the omentum to influence muscle tissue repair.

Consistent with the literature, we found the production of HGF and SDF-1 in both the omentum of mdx and C57BL/10 mice. These factors have putative roles in muscle biology with HGF stimulating satellite cell activation and proliferation but inhibiting myoblast fusion and terminal myogenic differentiation in vitro (Tatsumi et al. 1998; Witt et al. 2017). Moreover, these factors are upregulated in regenerating areas of mdx muscles and in human muscular lesions (Proto et al. 2015). SDF-1 is a stem cell chemotactic factor known to participate in muscle ontogenesis, regeneration and myoblast differentiation. Moreover, SDF-1 attracts hematopoietic and mesenchymal stem cells to the injury loci and improves the migration of transplanted myoblasts (Pituch-Noworolska et al. 2003; Odemis et al. 2007; Kowalski et al. 2016). Hence, we found increased VEGF expression in the omentum of mdx compared with that of the control mice. VEGF factor is a proangiogenic molecule with pleiotropic actions directly on muscle progenitors enhancing migration and preventing apoptosis, besides stimulating terminal muscle differentiation (Messina et al. 2007; Bryan et al. 2008). It is conceivable that increased VEGF levels in the omentum promote the migration of muscle progenitors into the diaphragm. VEGF, HGF and SDF-1 growth factors are mostly produced by myeloid cells and

especially macrophages and mast cells (Shaik-Dasthagirisaheb et al. 2013; Cavalla et al. 2013) in the immune aggregates and milk spots from the mdx omentum. Interestingly, omentum from mdx mice presents high numbers of mast cells at both 12 and 24 weeks of age (data not shown) suggesting that they may be the main producers of VEGF, HGF and SDF-1 growth factors in the mdx omentum.

Importantly, among all mdx skeletal muscles, the diaphragm presents the most peculiar pattern of regeneration and fibrosis with a constant production of inflammatory mediators: MMP-9, MMP-2; RANTES and MP1-α chemokines and receptors (Demoule et al. 2005; Bani et al. 2008). In addition, deposition of a high content of hydroxylated collagen I is a hallmark of the fibrotic repair present in the DMD diaphragm (Smith et al. 2016). Interestingly, omentectomy decreased collagen occupied areas in mdx diaphragm at both 12 and 24 weeks when compared with respective SHAM groups. Nevertheless, polarized light analysis revealed an increased proportion of collagen I deposition evidencing fibrotic remodeling at the expense of collagen IV reduction. As collagen IV is a major component of the basal lamina of myofibers and blood vessels (Roggendorf et al. 1988; Sanes 2003), we conclude that such reduction may be due to loss of structural integrity and reduction of the niche for satellite cells and vascular-associated progenitors. Likewise, collagen IV turnover dynamics can greatly impact the physiology of muscle regeneration, myoblast fusion and differentiation by affecting the availability of IGF-I (Ito et al. 2015).

Another factor that greatly impacts extracellular matrix remodeling in muscular lesions is macrophage infiltration. M-2 macrophages are pivotal players in muscle regeneration by enhancing membrane repair, inhibiting inflammation and oxidative stress and also producing growth factors and stimulating fibroblasts for proper extracellular matrix remodeling (Tidball and Wehling-Henricks 2007; Arnold et al. 2007; Villalta et al. 2015). Herein, we observed that omentectomized mice had marked reduction on the M-2 macrophage subset, an indication that omentectomy may be detrimental for the diaphragm remodeling profile. Another interesting aspect is that omentectomy markedly reduced NFκB expression, a transcription factor that regulates the inflammatory immune response and also expressed in muscle precursors with pro- and anti-myogenic effects depending upon the activation of the signaling pathway (Canicio et al. 2001; Bakkar et al. 2008; Bakkar and Guttridge 2010). Likewise, we found increased levels of TNF-α, a cytokine that can be produced by differentiating myoblasts; besides the inflammatory activity that further stimulates myoblast differentiation and fusion (Collins and Grounds 2001; Chen et al. 2007). Dual-knockout mice for TNFR1 and TNFR2 exhibit low expression of MyoD and MEF-2 after acute freezing injury, suggesting that TNF-α may promote early stages of the myoblast differentiation process, albeit with the maintenance of the compromised force recovery after injury (Warren et al. 2002). Such a result potentially corroborates with the enhanced levels of myogenin expression in OTX diaphragm. However, we did not observe an increase of regenerating myofibers in the omentectomized diaphragm by histological analysis probably due to a change in the profile of the macrophages in the OTX diaphragm.

The interaction between macrophages and satellite cells is essential for muscle fiber differentiation and defects in the regeneration program may be a consequence of such a compromised interaction (Kosmac et al. 2018). Cellular therapy is a promising approach in degenerative diseases like DMD; nevertheless, overcoming major short-comings like the low migration capacity of myoblasts, rejection of dystrophin transfected cells and inefficient induction of muscle differentiation has proven very challenging (Sienkiewicz et al. 2015; Siemionow et al. 2018). Efficient muscle repair requires an elaborated blend of environment factors derived from local and peripheral tissue associated with the inflammatory response (Dinulovic et al. 2017). The present study clearly indicates that the omentum is essential to maintain an appropriated environment for promoting the regeneration of the mdx diaphragm muscle.

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